

Study of Some Virulence Factors of *Proteus mirabilis* Isolated from Urinary Stones Patients

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Abstract

A total of 125 specimens of stones and urine were collected from urinary stone patients from (June to December, 2012). According to primary identification, which based on macroscopic and microscopic characteristics and biochemical tests, 25 (20%) and 100 (80%) of isolates were identified as *Proteus* and non-*Proteus*, respectively. The 25 *Proteus* isolates were finally identified as *Proteus mirabilis* based on Vitek 2 system and polymerase chain reaction (PCR) technique by using target gene 16S *rRNA*. The multiple logistic regressions results showed that the age > 40 years old was a risk factors that significantly associated with increased incidence of *P. mirabilis* in urinary stone patients, as $P = 0.02$, and Odd's ratio (OR) was 4.889 (1.7-14.057), while in relation to gender, the analysis revealed that they were statistically non-significant as OR was 1.174 (0.488-2.822) as well as $P=0.720$.

Some virulence factors of all isolates were investigated by Qualitative and Quantitative assay. Qualitative assay showed that all isolates (100%) were positive to urease, biofilm, Adhesion factors, and swarming activity. Whereas, 40% of isolates were positive to protease and ESBL, 96% and 76% of isolates were positive to agglutination and β -lactamase, respectively.

Quantitative assay revealed that all tested isolates were significantly differences $P < 0.05$ in the production of the tested virulence factors. The urease production activity range from 59 to 129 U/ml, whereas the protease production activity ranged from 2.5 to 8 U/ml; the mean of adhering to uroepithelial cell ranged from (20-45) bacteria/cell. The mean of optical density ranged from (0.028-0.33) at OD₆₃₀ and the percentages of biofilm activity were 60%, 24% and 16% as strong, moderate and weak biofilm, respectively. The mean of swarming growth activity of *P. mirabilis* isolates ranged from (3-67mm) and 40%, 32%, 8%, and 20% of isolates showed strong, moderate, weak, and very weak swarming activity, respectively.

Introduction

The formation of urinary stones in human body is a serious clinical problem that affects up to 20% of the population with recurrence after treatment on the level of 50% (Benramdane *et al.*, 2008). Urinary stones from in course of many physical and chemical processes, besides organic matrix, they consist mainly of crystalline phases of various substances and they are divided into two kinds. The first kind of urinary stones is related to metabolic disorders. In the case of such stones, the most predominant crystalline components are calcium oxalate monohydrate, carbonate apatite, and calcium oxalate dehydrates. Some of the components of metabolic stones are very easy to dissolve while; others may grow as crystals with sharp edges that may damage the patient's tissues (Prasongwatana *et al.*, 2008 and Ali Al-Marzoqi *et al.*, 2014).

The second kind of stones is called infectious, which is stones related to urinary tract infection (Bichler *et al.*, 2002). Some of these stones are revealing fractures of spherical structures, which probably are spherical particles of calcium carbonate (Martel and Young, 2008). These structures are morphologically similar to bacteria and taking into account the size of these particles, they may take some kind of coccoid-shape urinary pathogens (Kontoyannis and Vagenas, 2000).

The most commonly encountered bacteria are *Proteus mirabilis*, *Proteus vulgaris*, *Ureaplasma urealyticum*, and *Staphylococcus aureus*. They are mainly the microorganisms producing urease enzyme, the microorganism from the species *Proteus* are isolated in the case of 70% of the so-called infectious stones (Kramer *et al.*, 2000).

Proteus mirabilis is a gram-negative enteric bacterium that occurs as vegetative swimmer cells and hyperflagellated swarmer cells (Rozalski *et al.*, 2012). Individuals suffering from urinary tract infections (UTIs) caused by *Proteus mirabilis* often develop bacteriuria, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever (Sosa and Zunino, 2010). Moreover, *Proteus mirabilis* is one of the most common causes of UTIs in individuals with long-term indwelling catheters, complicated UTI, and bacteremia among the elderly (Jacobsen *et al.*, 2008). As the aging population expands, more individuals will be at risk for *Proteus mirabilis* UTIs and stones formation (Burall *et al.*, 2004).

Potential virulence factors and bacterial behaviors associated with the infection processes and disease, including swarming, growth rates, fimbria expression, flagella, and the production of hemolysins, ureases,

proteases, amino acid deaminases, in addition to the expression of lipopolysaccharide (LPS) antigens and capsular polysaccharides (CPSs), have been described in many studies (Jacobsen *et al.*, 2008 and Al-Marzoqi *et al.*, 2013). Both LPSs and CPSs have been considered to play an important role in the progression of UTIs, in addition to affecting both kidney and bladder stone formation (Torzewska *et al.*, 2003 and Ali H. Al-Marzoqi *et al.*, 2009). Furthermore, the LPS O antigen confers protection against serum-mediated bactericidal activity (Kaca *et al.*, 2009; Rozalski, 2008, Ali Al-Marzoqi *et al.* 2014), and bacterial LPS released from bacteria is a biologically active endotoxin that causes a broad spectrum of pathophysiological conditions, including septic shock (Raetz and Whitfield, 2002). Recently, two additional virulence factors with cytotoxic and agglutination properties, the high-affinity phosphate transporter (Pst) and the autotransporter (Pta), have been described (Alamuri and Mobley, 2008; Jacobsen *et al.*, 2008).

Materials and Methods

Patients

A total of 125 specimens were collected from patients suffering from urinary stone disease (61 male and 64 female with age ranged between 16-65 years old) who were admitted to three Hospitals: Lithotripsy Center in Al-Hilla Educational Hospital, Al-Qasim Hospital and Al-Hashymia Hospital to the period 6/2012 to 12/2012. The specimens were included 25 stones specimens and 100 urine specimens.

Collection and examination of specimens

A total of 25 stone specimens of suitable size were collected in sterilized containers and taken to the Department of Microbiology in the College of Veterinary Medicine for bacterial analysis. Stones were washed in sterile saline and crushed under aseptic conditions, then cultured in nutrient broth (Nemoy and Stanley, 1971). After overnight incubation at 37 °C, they were sub-cultured on MacConkey agar, blood agar, and nutrient agar and incubated at 37 °C for 24-48 hr. Colonies growing on MacConkey agar were considered Gram-negative bacteria (Collee *et al.*, 1996).

Mid-stream urine of 100 specimens was collected in sterilized screw-cap containers. Urine specimens were centrifugated at 10,000 rpm for 15 minutes. The supernant was discarded and sediments were cultured in MacConkey broth and incubated at 37°C for 24 h, followed by streaking on MacConkey agar, blood agar and nutrient agar then incubated at 37°C for 24 h. (Collee *et al.*, 1996).

Laboratory diagnosis

The laboratory diagnosis of isolates was included colony morphology and microscopic examination according to MacFaddin (2000). The biochemical tests were done to differentiate the bacterial isolates, which included urease test according to Benson (1998), while H₂S production, motility, and indole production test has been done according to MacFaddin (2000).

Vitek 2 System for identification of bacteria

The bacterial isolates were identified using Vitek 2 System. The Vitek 2 system identifies bacteria and other microorganism based upon analysis of substrate utilization patterns. Selection of cards to be used depended upon the gram stain results and growth conditions of the organism to be tested (Pincus, 2010).

Confirmative identification of *P. mirabilis* isolates by PCR

PCR assay was done according to methods described by (Lu *et al.* 2000) for detection of 16S *rRNA* gene of *P. mirabilis* isolates as follows:

Genomic DNA extraction:

Genomic DNA of *P. mirabilis* isolates was extracted by using Genomic DNA Mini Kit, using manufacture's protocol. The concentration and purity of extracted DNA were measured using Nanodrop spectrophotometer at absorbance (260/280 nm) at ratio 1.8 as pure DNA, and done according to Desjardins and Conklin (2010).

Primers and PCR conditions:

The DNA amplified with primer sequencings (5-CATGTGTAGCGGTGAATG-3), (5-GTAAGGGCCATGATGACTT-3). Polymerase chain reaction master mix reaction was prepared by using AccuPower PCR PreMix Kit and this master mix was done according to company instructions as follows:

PCR Master mix reaction components		Volume
PCR PreMix (Lyophilized)		5 µl
DNA template		5 µl
Primers	F. primer	1.5 µl
	R. primer	1.5 µl
PCR water		7 µl
Total volume		20 µl

PCR thermocycler conditions for 16S *rRNA* gene were done by using convectional PCR thermocycler system as follows:

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	5sec.
Annealing		55C	30sec
Extension		72C	45sec
Final extension	1	72C	7min
Hold	-	4C	Forever

Detection of PCR product:

The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 7 µl of the final reaction mixture in 1.5% agarose and the gel was then destained under running water for 10 min. 100 bp DNA ladder (Gene dire) was used as molecular markers (Sambrook and Russell, 2001).

Qualitative and quantitative virulence factors assay

Qualitative and quantitative assay of virulence factors of *P. mirabilis* isolates were investigated for urease, adhesion, biofilm, swarming, protease, β-lactamase and ESBL according to the reference method for each assay as follows:

Type of assay	Reference method	
	Qualitative	Quantitative
Urease	MacFaddin, 2000	Weatherburn, 1967
Adhesion	Andreu <i>et al.</i> , 1995	Andreu <i>et al.</i> , 1995
Biofilm	Christensen <i>et al.</i> , 1982	Stepanovic <i>et al.</i> (2007)
Swarming	Kwil <i>et al.</i> , 2013	Kwil <i>et al.</i> , 2013
Protease	Wassif <i>et al.</i> , 1995	Ayora <i>et al.</i> , 1994
Agglutination	Evans <i>et al.</i> , 1979	-
β-lactamase	Catlin, 1975	-
ESBL	Jarlier <i>et al.</i> , 1988	-

Results and Discussion

Isolation of *P. mirabilis*

A total of 125 specimens representing patients clinically diagnosed as urinary stone patients distributed as 61 specimens from males and 64 specimens from females. These specimens yielded 25 *Proteus* isolates in percentage 20% as shown in the table (1). These results were in agreement with Al-Bassam and Al-Kazaz (2013) as they found that the percentage of isolated *P. mirabilis* was 19.04%. This result was similar with the results of Pitout *et al.* (1998), they isolated *Proteus* in 20 %of cases. Also, this result was parallel with Nass *et al.* (2001) they were isolated *P. mirabilis* from renal calculi in percentage 21%.

For determining the risk factors which were associated with increased incidence of *P.mirabilis* form urinary stone patients, multiple logistic regression analysis was done, and the analysis revealed that only the age was considered as a risk factor where the Odd's Ratio (OR) was 4.88, 95% Confidence Interval (CI) was (1.7-14.057), and *P* value was 0.02. These results were in agreement with Jroundi *et al.* (2007), their results were found that the risk factors for development of *P.mirabilis* nosocomial infection, were age OR was 1.09, CI (0.60-2), as well as other factors they were investigated such as linked to surgical category, a hospital stay of more than 10 days, and the use of intravascular and urinary devices. The current results were in agreement with Guerado *et al.* (2010), they found that only age increases the risk factor for patients with nosocomial infection, the OR value was 0.79 and CI value was (0.41-1.53).

On recording the relation between *P.mirabilis* infections and gender, it was found that the distribution in both males and females was not statistically significant *P* value = 0.720. This result was similar with (Guerado *et al.* 2010 and Ali H. Al-Marzoqi, 2008), they reported that the gender was not associated with a higher risk of developing of *Proteus* spp. nosocomial infection.

Table (1): The risk factors associated with urinary stone patients infected with *Proteus* spp.

Variables	Groups	Total cases	<i>Proteus</i> spp.		Non- <i>Proteus</i> spp.		Odd's Ratio(OR) (95% CI)	<i>P</i> value
			No	%	No	%		
Age	>40	65	20	30.76	45	69.23	4.889 (1.7-14.057)	0.02
	≤40	60	5	8.33	55	91.66		
Gender	Male	61	13	21.31	48	78.68	1.174 (0.488-2.822)	0.720
	female	64	12	18.75	52	81.25		
Total		125	25	20	100	80		

Identification of *Proteus mirabilis*:

Colonies of *P. mirabilis* on MacConkey agar appeared pale, yellow, and non-lactose fermenters; these results were similar to Winn *et al.* (2006). While on blood agar *P. mirabilis* isolates showed the swarming phenomenon as well as the fish odor. On nutrient agar, also, *P. mirabilis* show the swarming phenomenon and they were colorless, these results were in agreement with Janda and Abbott (2006) and Hawkey (2006). The microscopic examination of *P. mirabilis* showed that bacteria were Gram negative, appeared as small cocci while some of them appeared as long filaments cocci, the same results had been done by Dennis (2001). The results of biochemical tests of *P. mirabilis* isolates showed positive results to product urease enzyme, motion test and H₂S production test, while negative result for indole test in SIM media. These results were identical with the studies of Manos and Belas (2006) and Hawkey (2006).

The results of identification of *P. mirabilis* using Vitek system table (2) showed all isolates were *P. mirabilis* and the percentage of identification was ranged from (95-99%), and this percentage was in agreement with Bourbeau and Heiter (1998) as they reported the identification of *Proteus* spp. by Vitek 2 system was in 97% percentage.

Table (2): The results of identification of *Proteus mirabilis* isolates by using Vitek 2 system.

No. of well	Symbol of test	Result	No. of well	Symbol of test	Result
2	APP A	-	33	SAC	-
3	ADO	-	34	dTAG	-
4	PyrA	-	35	dTRE	+
5	IARL	-	36	CIT	+
7	dCEL	-	37	MNT	-
9	BGAL	-	39	5KG	-
10	H ₂ S	+	40	ILATK	-
11	BNAG	-	41	AGLU	-
12	AGLTP	-	42	SUCT	+
13	dGLU	+	43	NAGA	-
14	GGT	+	44	AGAL	-
15	OFF	+	45	PHOS	+
17	BGLU	-	46	GlyA	-
18	dMAL	-	47	ODC	+
19	dMAN	-	48	LDC	-
20	dMNE	-	53	IHISa	-
21	BXYL	-	56	CMT	+
22	BAlap	-	57	BGUR	-
23	ProA	-	58	O129R	+
26	LIP	-	59	GGAA	-
27	PLE	-	61	IMLTa	-
29	TyrA	+	62	ELLM	-
31	URE	+	64	ILATa	-
32	dSOR	-			

The results of PCR identification of *P. mirabilis* using 16S *rRNA* showed all isolates were *P. mirabilis* and gave a good confirmative identification as shown in the figure (1). All *P. mirabilis* isolates yielded the same band size (538bp) which was the product size of primers used for identification. This result was in agreement with the study of Schabereiter-Gurtner *et al.* (2001) they used 16S *rRNA* to identify *Proteus* spp. pathogens in monomicrobial and polymicrobial ocular infections and the study of Lu *et al.* (2000), they used 16S *rRNA* for detection *Proteus* spp. pathogens in cerebrospinal fluid (CSF). The two above study were reported that 16S *rRNA* was a high discriminatory power for identification of *Proteus* spp..

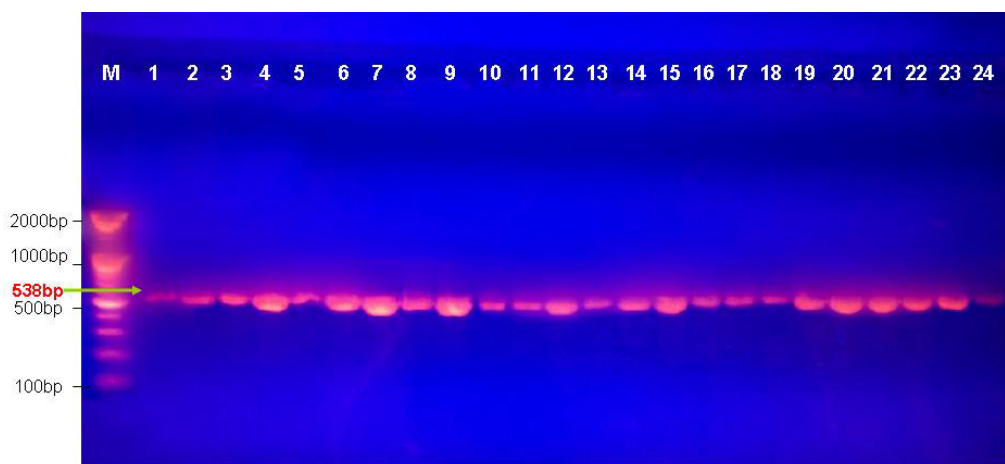


Figure (1): The confirmative identification of *P. mirabilis* based on 16S *rRNA* by conventional PCR method. M: 100 bp DNA ladder, Numbers (1-24): *P. mirabilis* isolates number.

Virulence factors

Qualitative assay

Some virulence factors of 25 *P. mirabilis* isolated from urinary stone patients were tested as shown in the table (3), the results revealed that all 25 isolates were positive for urease production, biofilm formation, adhesion test and swarming with 100%. These results were in agreement with the study of Al-Duliami *et al.* (2011), they reported that all *Proteus* spp. were produced urease enzyme in 100%. The results agree with other studies, which found that *P. mirabilis* isolates were able to produce biofilm (Al-Ouqaili and Al-Kubaisy, 2008). The studies of Rozalski *et al.* (2012) documented a high ability of *P. mirabilis* for adhesion to epithelial cells and attributed to the presence of cilia and pili of these microorganisms. The studies of Belas (1992) showed that *P. mirabilis* and other *Proteus* spp. have the ability to swarm on different swarming agar media.

For agglutination activity of human RBCs and protease production, the results showed that 24 and 11 isolates were positive for these two tests with percentages 96% and 44%, respectively. This result was matched with the result of Al-Duliami *et al.* (2011) as they reported that 95.8% of *Proteus* spp. were positive to agglutination test. Also, this results were in agreement with the study of Al-Salihi (2012), she reported that *P. mirabilis* isolates produced protease in percentage 41.9%. Al-Duliami *et al.* (2011), as they showed that 11 isolates of *P. mirabilis* and *P. vulgaris* were able to produce protease in percentage 45%.

On the other hand, there were 19 and 10 isolates explained positive results for β -lactamase and extended β -lactamase with percentages 76% and 40%, respectively. This result was in agreement with Feglo *et al.* (2010) who found that *P. mirabilis*, *P. penneri*, and *P. vulgaris* isolates were produced β -lactamase in percentages 77%, 75%, and 65.4 respectively. Pagani *et al.* (2002) who stated 40% of *P. mirabilis* isolates were produced ESBL. Tonkic *et al.* (2010) reported that the prevalence of ESBLs in *P. mirabilis* isolated from southern Croatia was increased from 0.5 % in 2005 to 20.9 % by 2008.

Table (3): The percentages of some virulence factors of *P. mirabilis* isolated from urinary stone patients.

Virulence factor	Positive		Negative	
	No.	%	No.	%
Urease production	25	100	0	0
Biofilm formation	25	100	0	0
Adhesion	25	100	0	0
Swarming	25	100	0	0
Agglutination	24	96	1	4
Protease production	10	40	15	60
β -lactamase	19	76	6	24
ESBL	10	40	15	60

Quantitative assay:

The results of quantitative urease assay were shown in the figure (2) revealed that urease production activities of isolated *P. mirabilis* were ranged from (59-126) U/ml. These results were in agreement with Al-Baghdady *et al.* (2009) as they reported that the urease activity of *P. mirabilis* and *P. penneri* was ranged from (59-129) U/ml. The present study showed there were significant differences ($P < 0.05$) among *P. mirabilis* isolates, and this was similar to the study of Stankowska *et al.* (2008), they reported that 12 of *P. mirabilis* isolates were showed significant differences and they divided these isolates into high ureolytic group and low ureolytic group.

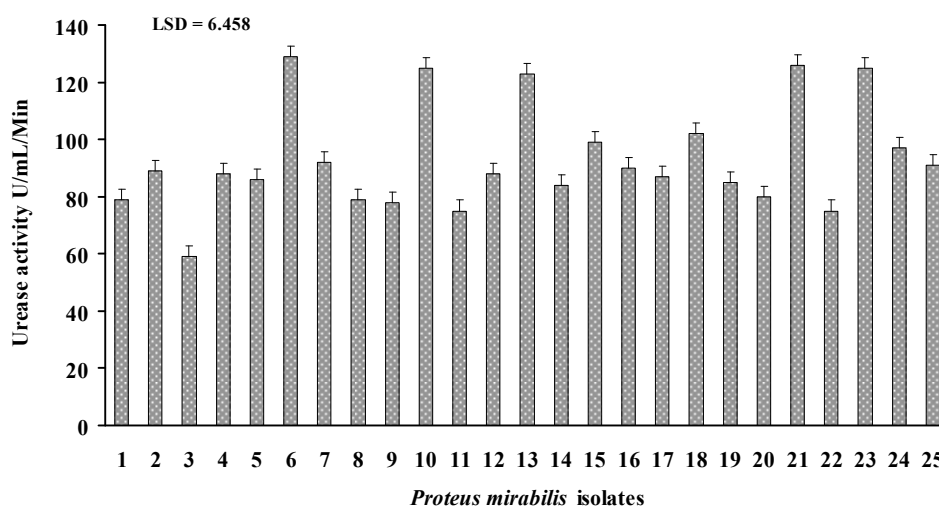


Figure (2): Ureolytic activity of *P. mirabilis* isolates from urinary stone patients. Bars indicate to standard error.

The quantitative results of adhesion assay were revealed that high mean number of adhesion was 45 (bacteria/cell) for isolate number 6 as shown in figure (3). This results was in agreement with Al-Baghdady *et al.* (2009) they found that the highest mean number of adhesion was 45 (bacteria/cell) for the *P. mirabilis* number 12, and also, this result almost in agreement with that recorded by Al-Shebani and Al-Jeboury (2008) they showed that the highest number of adhering bacteria to uroepithelial cell ranged from (45-55) bacteria/ cell by the isolate *P. mirabilis* number 9.

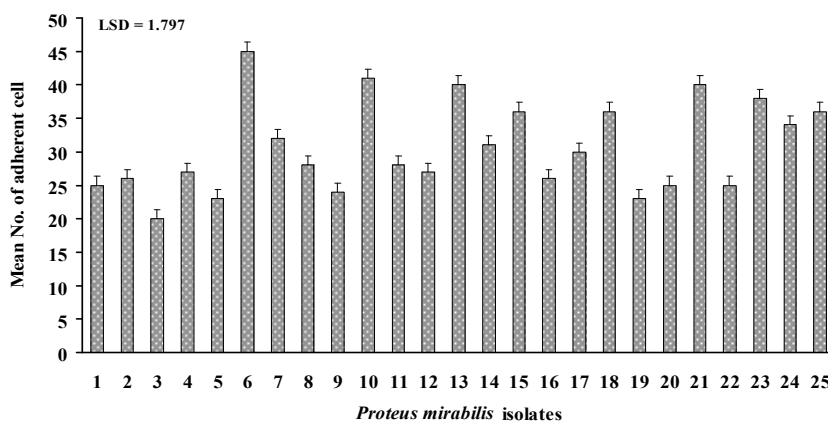


Figure (3): The adhesive capacity of *P. mirabilis* isolated from urinary stone patients. Bars indicate standard error.

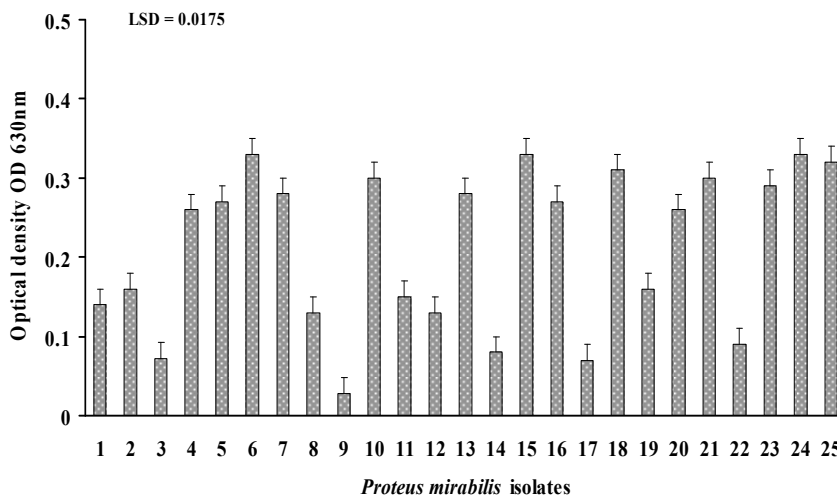


Figure (4): The mean of optical density of Biofilm formation assay of *P. mirabilis* isolates from urinary stone patients. Bars indicate standard error.

The quantitative biofilm assay results were showed significantly differences among *P. mirabilis* isolates (OD₆₃₀) as shown in the figure (4). These results were parallel to the results of Marinela *et al.* (2012) they recorded differences among *Proteus* spp. at OD₆₃₀. The present results showed that the highest biofilm formation was 0.33 at OD₆₃₀ and this was in agreement with Al-Ouqaili and Al-Kubaisy (2008) as they recorded that all *Proteus* spp. were produced biofilm strongly (OD was more than 0.25). The results of current study were categorized the biofilm formation of *P. mirabilis* isolates as strong, moderate, and weak biofilm. A strong biofilm was formed by 15 (60%), moderate biofilm by 6 (24%), and weak biofilm by 4 (16%) as shown in figure (5). These results were nearest from the results of Kwiecinska-Pirog *et al.* (2013), they examined 50 *P. mirabilis* isolates for biofilm formation and found that weak biofilm was formed by 12 (24%), moderate by 13 (26%), and strong by 25 (50%).

Strong biofilm forming was confirmed for 14 (56.0%) strains isolated from urine and 11 (44.0%) for strains isolated from wound swabs (Kwiecinska-Pirog *et al.*, 2013).

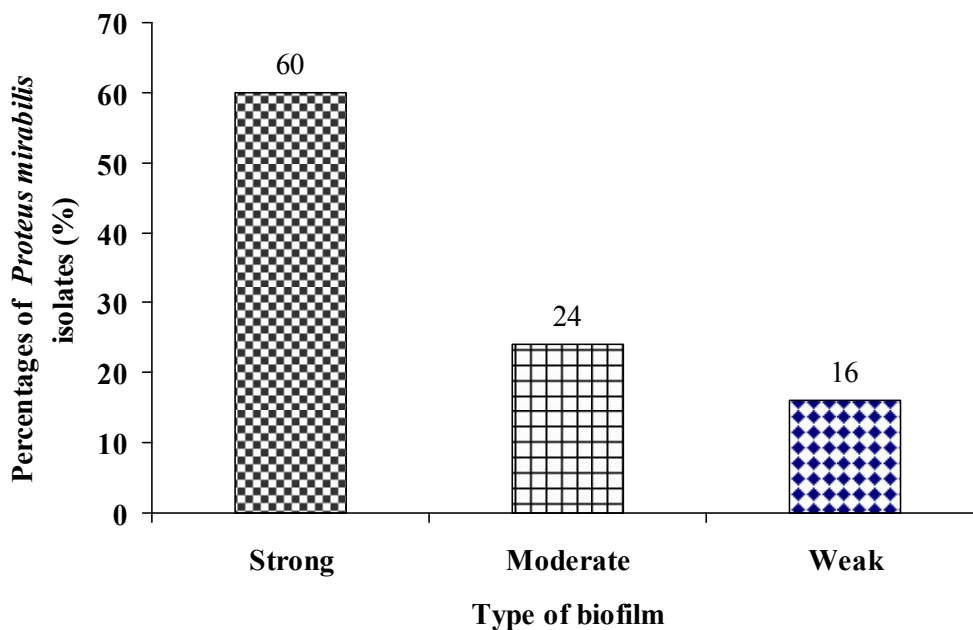


Figure (5): The percentages of biofilm formation assay of *P. mirabilis* isolates from urinary stone patients.

The results of swarming assay showed that the swarming growth ranged from (3-67 mm) and the highest swarming growth activity was 67 mm for *P. mirabilis* isolate number 6, while the lowest swarming growth activity was for isolate number 3 and 8 (3 mm) as shown in the figure (6).

These results were in parallel with the study of Chouduri *et al.* (2014) who showed that the *P. mirabilis* isolates recorded the highest swarming activity (70.67 mm) compared with *P. vulgaris* and *P. penneri* isolates. Kwil *et al.* (2013) showed the highest swarming growth after 24 hr was 67 mm for *P. penneri* isolate number 54, while the lowest swarming growth was two mm for *P. penneri* isolate number 61, these results were in agreement with our results.

The figure (7) showed that five isolates (20%) were very weak swarming, two isolates (8%) were weak swarming, eight isolates (32%) were moderate swarming, and 10 isolates (40%) were strong swarming. These results were in converged to that of Kwil *et al.* (2013), who showed that 17 isolates (24%), six isolates (8%), 22 isolates (31%), 26 isolates (36%) of *P. penneri* were very weak, weak, moderate, and strong swarming ability, respectively.

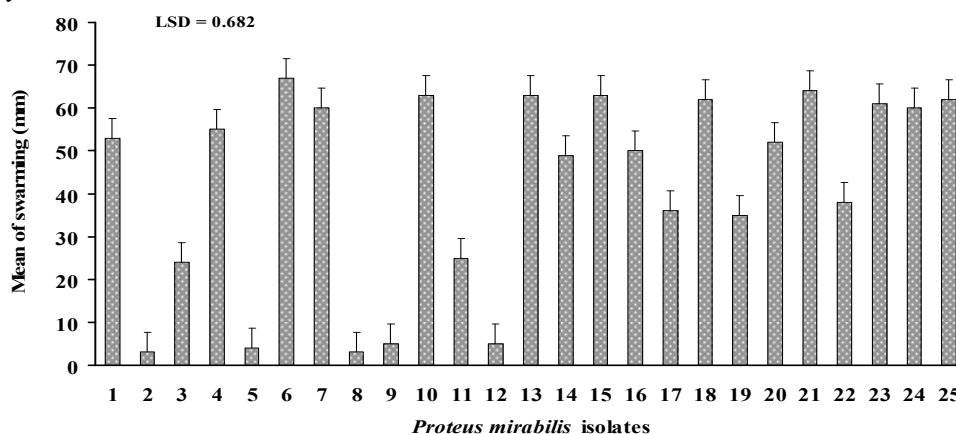


Figure (6): Swarming motility assay of *P. mirabilis* isolates from urinary stone patients calculated after 24 h of incubation. Bars indicate standard error.

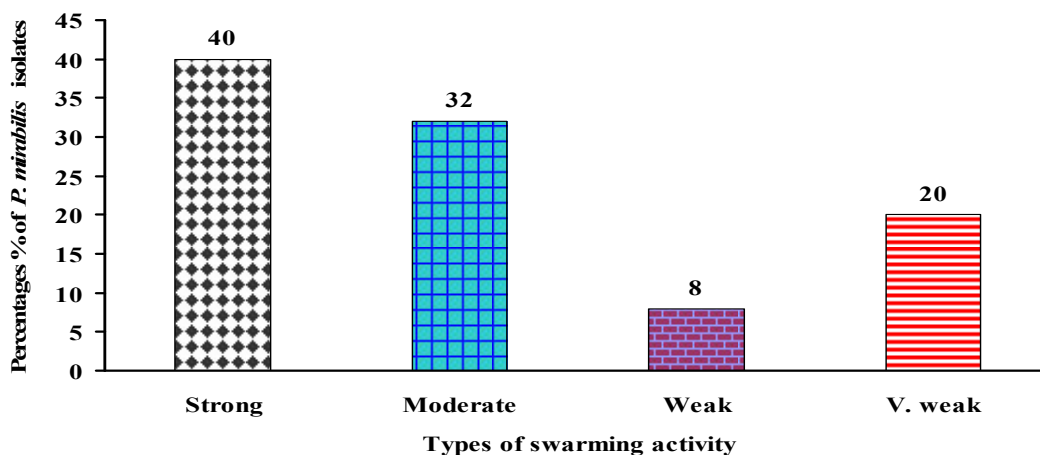


Figure (7): Percentages of swarming assay of *P. mirabilis* isolates from urinary stone patients.

The quantitative results of protease assay were showed that the protease production of the tested isolates ranged from (2.5-8 U/ml) and the highest protease activity was 8 U/ml for isolate number 6, whereas the lowest protease activity was 2.5 U/ml for isolate number 3 as shown in figure (8). These results were in agreement with Prakash *et al.* (2011) who recorded the highest protease activity (8 U/ml) of *P. mirabilis* strains as well as the protease activity was ranged from (0.133-8 U/ml) and also, he recorded the highest protease activity for *P. vulgaris* (3.5 U/ml), while the lowest activity for *Enterobacter aerogenes* was 2.133 U/ml. The current results showed there were statistically differences among *P. mirabilis* isolates $P < 0.05$ and these result was similar with the result of Stankowska *et al.* (2008), as they reported a statistically differences in proteolytic activity among *P. mirabilis* strains. Stankowska *et al.* (2008) showed that protease activity was ranged from (4.9-6.8) U/ml and this result had a slight differences compared with the results of the present study.

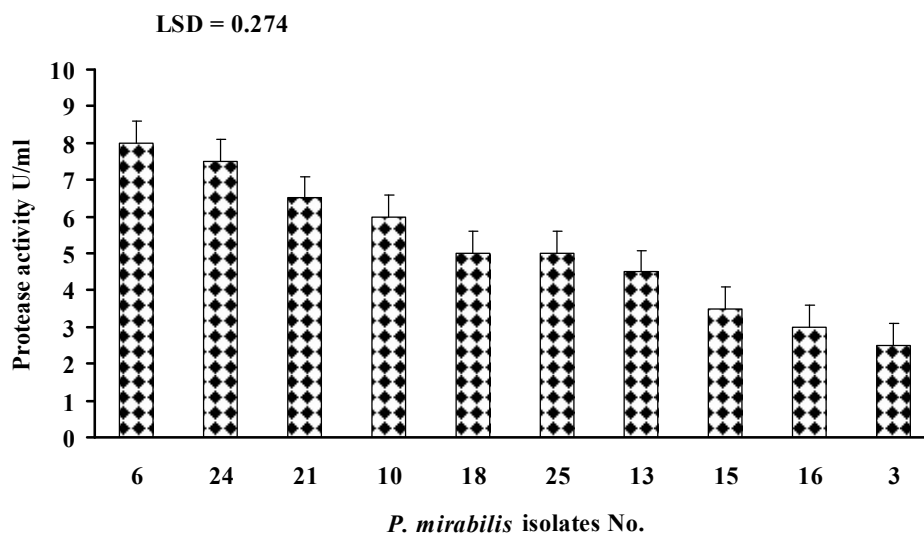


Figure (8): Proteolytic activity of *P. mirabilis* isolated from urinary stone patients. Bars indicate standard error.

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